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Optical tweezers-based immunosensor detects femtomolar concentrations of antigens

KRISTIAN HELMERSON,[1]* RANI KISHORE,[1] WILLIAM D. PHILLIPS,[1] and HOWARD H. WEETALL[2]

We used optical tweezers (optical trapping technology) to measure the force required to separate antigen–antibody bonds. Under competitive-binding conditions, we used the force determination to detect and measure protein antigen concentrations as small as 1 fmol/L (10^{-15} mol/L).

INDEXING TERMS: lasers - antigen–antibody binding • immunoassays

Detection and quantification of ultralow concentrations of analytes are becoming increasingly important *[1]*. Methods that do not require amplification techniques to detect infectious organisms, viruses, and nucleic acid targets could find wide application in the clinical environment. To this end, we have carried out a series of experiments leading to the detection of femtomolar concentrations of a protein antigen by a method we believe could be widely applicable and capable of automation. This approach involves optical trapping technology capable of sensing single antigen–antibody bonds. Using a competitive-binding or displacement-type assay, we can detect extremely small quantities of a soluble antigen added to the system.

Materials and Methods

PRINCIPLE

We have constructed a sensor that is based on optical trapping technology, i.e., optical tweezers. Optical tweezers are focused laser beams used to trap and remotely manipulate dielectric particles, including cells and other biological objects *[2–4]*. The change in momentum of the light transmitted by the dielectric object results in a force that traps objects having an index of refraction greater than that of the surrounding medium at the local maximum of the intensity of the electromagnetic field, i.e., at the focus of the laser beam. Svoboda and Block *[4]* have reviewed the principles of optical forces as well as the various configurations and applications of optical tweezers.

Figure 1 illustrates the basic principle of our device. We use optical tweezers to trap a microsphere coated with an antigen and then pull the microsphere away from a surface coated with the corresponding antibody. Throughout, we measure the force applied by the optical tweezers to break the antigen–antibody bonds and to pull the microsphere away from the surface. To detect the presence of small quantities of the antigen in solution, we use a competitive-binding displacement approach: The binding of the free antigens in solution to the antibodies on the surface is detected as a decrease in the average force required to pull the antigen-coated microsphere away from the surface.

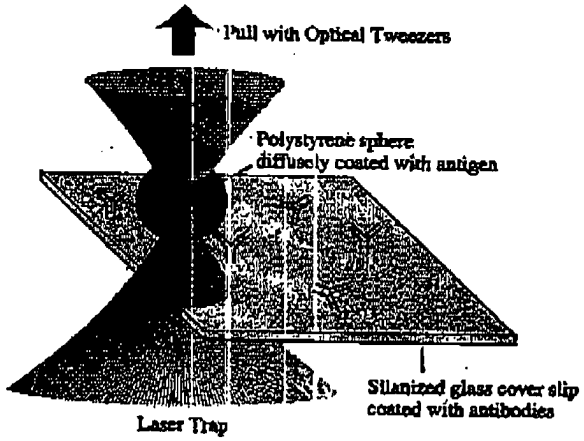

Fig. 1. Basic scheme of our optical tweezer-based immunosensor.
An antigen-coated microsphere is trapped and pulled away from an antibody-coated surface by use of the optical tweezers. The minimum amount of force applied by the tweezers to break the microsphere-coupled antigen–antibody bonds is measured. Detection of free antigens in solution is manifested as a reduction of this applied force, the result of displacement of microsphere-coupled antigen by free antigen in the binding to the antibody.

[1] Atomic Physics Division, and [2] Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD 20899.
*Author for correspondence. Fax 301-975-3038; e-mail kristian@enh.nist.gov.
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APPARATUS[3]

Our optical tweezers apparatus consists of a Zeiss inverted microscope equipped with a 100× (numerical aperture 1.4) oil-immersion objective lens, a video camera and monitor, a computer-driven translation stage capable of motion in three orthogonal directions, and a continuous-wave Nd:YAG laser emitting light at a wavelength of 1.06 μm. The laser light is coupled into the back of the objective lens with a dichroic mirror, which enables us to simultaneously view and trap the microspheres. Transparent, polarizable objects with an index of refraction higher than the surrounding medium, such as these microspheres in buffer, are trapped at the local maximum of the intensity of the laser light, tightly focused by the objective lens. Typically, the size of the focal spot is kept fixed; therefore, the strength of the trapping force is proportional to the power of the laser.

The microspheres, suspended in a buffer solution, were contained in a chamber constructed from a glass microscope slide with a 1.0-cm hole drilled through it and two glass cover slips on each side. The coverslip on the objective-lens side of the chamber contained the silane-coupled antibodies. Both cover slips were sealed to the microscope slide with silicone vacuum grease. The total volume of the chamber was ~100 μL.

SAMPLE PREPARATION

The antigen used in these experiments, bovine serum albumin (BSA, 98-99%; Sigma Chemical Co., St. Louis, MO), was covalently coupled to 4.5-μm-diameter latex microspheres with carboxyl groups (Bangs Labs., Carmel, IN). The covalent coupling was accomplished by first removing 0.1 mL of the original 50 g/L suspension of microspheres and washing with pH 6.6 buffer (0.05 mol/L potassium phosphate, 0.1 mol/L sodium chloride, 2 g/L gelatin, and 0.1 mL/L thimerosal). Next, we added 1.0 mL of 50 mmol/L 2-(*N*-morpholino)ethanesulfonic acid buffer (Sigma Chemical Co.), pH 5.5, to the desired quantity of BSA plus microspheres and vortex-mixed. A freshly prepared 10 g/L solution of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma Chemical Co.) was added to the microspheres and stirred for 4 h. Unreacted carbodiimide was removed by centrifuging and washing the microspheres three times at 15 °C with phosphate-buffered saline (PBS; 0.05 mol/L potassium phosphate and 0.1 mol/L sodium chloride), pH 7.5, containing 0.1 g/L thimerosal. We stored the washed microspheres in PBS at 4 °C. For the experiments reported here, we diluted the BSA-coupled microspheres to ~0.1 μmol/L, which yielded ~1000 microspheres in the sample chamber.

[3] Certain commercial materials and products are identified in this paper to adequately specify the experimental procedures. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology.

The corresponding antibodies, mouse monoclonal anti-BSA antibodies, were covalently attached to glass cover slips through silane coupling. Silane-coated cover slips were prepared by first boiling the cover slips in 10% nitric acid for 1 h and then washing with distilled water until the pH of the water was neutral. Silane solution was prepared by adding 5 mL of 3-glycidoxypropyltrimethoxysilane and 5 mL of tetramethylorthosilicate (both from Aldrich Chemical Co., Milwaukee, WI) to 100 mL of deionized water. The pH of the silane solution was adjusted to 4.0 with 10% acetic acid solution. The cover slips were dipped in the silane solution, dried at room temperature, heated for 90 min in an oven at 110 °C, and then mounted on glass slides. To the coverslip surface we added 100 μL of 0.05 mol/L potassium phosphate, pH 8.0, followed by 10 μL of 2.8 g/L anti-BSA monoclonal antibodies (Sigma Chemical Co.). After incubating the slides at 5 °C for 72 h, we rinsed the coverslips with the PBS–thimerosal solution.

MEASUREMENT OF BINDING

We detected the binding of the microsphere to the coverslip surface as follows. Microspheres resting on the surface of the coverslip were located optically with the microscope. We focused the microscope objective on the surface of the coverslip and then positioned the objective in the center of the microsphere in the plane of the coverslip. Using the computer-driven stage, we displaced the objective lens 5.0 μm toward the coverslip surface. This corresponds to placing the focus of the laser beam about one microsphere radius above the middle of the microsphere, into the chamber, away from the coverslip surface. We then slowly increased the laser power from zero until the point at which the microsphere could be seen to jump away from the coverslip surface into the focus of the laser beam. The minimum power at which the microsphere was pulled into the optical trap was recorded. The laser power was typically increased over a timespan of 5 s. However, the minimum laser power required to lift the microsphere off the surface and into the trap was unchanged when we increased or decreased this interval twofold (data not shown).

Results and Discussion

SPECIFIC VS NONSPECIFIC BINDING EXPERIMENT

We performed an initial series of experiments to study specific and nonspecific binding of antigens to the silanized surfaces, i.e., with and without antibodies, respectively. Two series of measurements were made, involving microspheres coated with BSA and silane-coated cover slips with and without anti-BSA coupled to them. For each series of measurements, we varied the surface coverage of the microspheres by changing the amount of BSA offered during the coupling procedure (from 1.45×10^{-7} mol/L to 1.45×10^{-15} mol/L). Fig. 2 presents the titration data for the BSA-coated microspheres, i.e., the laser power required to pull a microsphere, coated at a particular

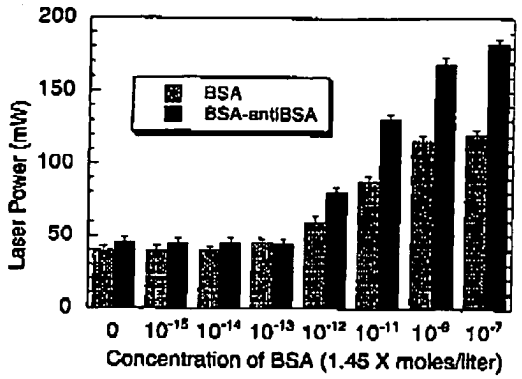

Fig. 2. Power required to pull microspheres coated with BSA off of a silane-coated glass coverslip (*light bars*) and off of a silane-coupled anti-BSA-coated coverslip (*dark bars*).

The increase in the measured laser power corresponds to increasing binding force between the BSA-coated microspheres and the silanized surface. The increase in the binding force with increasing BSA on the surface of the microspheres is interpreted as arising from the increasing number of BSA—anti-BSA bonds and of BSA–methyl-terminated silane bonds for the anti-BSA-coupled and noncoupled silane-coated surfaces, respectively. The BSA concentrations indicated are those used in the coupling reactions and not the quantities bound; therefore, they represent the maximum quantity of protein that could be coupled to the microspheres. The mean and SD of 10 measurements made with independent microspheres are shown.

concentration of BSA, off the coverslip surface (mean ± SD, n = 10).

As Fig. 2 shows, microspheres treated with BSA concentrations of 1.45×10^{-13} mol/L (0.145 pmol/L) or less bind to the coverslip surface with a force essentially the same as when there is no BSA on the microsphere. For microspheres coupled at 1.45 pmol/L or greater BSA concentrations, the binding force of the microsphere to the silanized surface increases with increasing BSA concentrations; however, the measured binding force of BSA to anti-BSA is much larger than the measured binding force of BSA to a coverslip coated with silane only. We interpret this as arising from both the specific and nonspecific binding of the BSA to the silanized surfaces with and without anti-BSA, respectively.

Given the number of microspheres used in the coupling reaction, and assuming 100% coupling of available BSA molecules to the microspheres, we calculate that the average number of BSA molecules coupled to the microspheres at the BSA concentration of 0.145 pmol/L is 1. (This estimate of the number of BSA molecules coupled to the microsphere is probably good within an order of magnitude. In practice, there will be <100% coupling of the BSA to the microspheres; however, loss of microspheres during washing will tend to compensate for the reduced coupling.) Thus, we expect that microspheres treated at BSA concentrations <0.145 pmol/L should have essentially no BSA available to bind to the coverslip surface. As the number of BSA molecules on the microsphere increases, the number of BSA-to-surface bonds for both specific and nonspecific binding should increase, along with a corresponding increase in the measured binding force.

The high selectivity provided by the molecular recognition of antibodies for antigens is shown in the data for Fig. 2. In each case in which microsphere-coupled BSA exhibited binding, the binding force of the BSA to a silane-coupled anti-BSA surface was larger than the binding force of the BSA to the silane surface without anti-BSA. Despite the strong tendency of BSA to bind to surfaces terminated with methyl groups (e.g., our nonfunctionalized silane surface *[5]*), the measured binding force of BSA to anti-BSA was 30% to 50% larger for microspheres containing coupled BSA at concentrations of 1.45×10^{-12} mol/L to 1.45×10^{-7} mol/L, respectively. Thus, Fig. 2 suggests that the increase in the specific binding of a microsphere coupled with BSA to the anti-BSA-coated silane surface occurs for microspheres coupled at BSA concentrations between 1.45×10^{-13} and 1.45×10^{-12} mol/L. In this range, the measured increase in the binding force would arise from a single antigen–antibody binding pair or at most a few such pairs.

In an additional experiment we tested our conclusion that the increased force observed with the BSA-coupled microspheres on the anti-BSA-coated coverslip was attributable to specific binding and not to the presence of any nonspecific IgG–BSA interaction. Because mouse IgG recognizes mouse serum albumin and does not cross-react with BSA, we coupled nonspecific mouse IgG (Sigma Chemical Co.) to a silanized coverslip and repeated the binding force measurement experiments with microspheres prepared at BSA concentrations ranging from 1.45×10^{-7} to 1.45×10^{-15} mol/L. The results showed that the force required to break the microsphere-to-surface bonds remained constant across the entire range of BSA concentrations coupled to the microspheres. This is consistent with the interpretation that the increased binding force observed in the presence of the specific antibodies results from the specific antigen–antibody interaction. In contrast with the data of Fig. 2, the absence of any increase in the nonspecific binding force of the BSA-coated microsphere with the silanized coverslip as the coverage of the microsphere increased suggests that the coverslip was, effectively, fully coated with the nonspecific mouse IgG. This would correspond to an average spacing between nonspecific IgG molecules of, at most, the radius of the microsphere. Hence, the minimum average surface density of nonspecific IgG is 2×10^7 molecules/cm^2.

IMMUNOSENSOR EXPERIMENT

To demonstrate use of the device as an immunosensor, we performed a typical competitive or displacement-type assay, using a BSA-coated microsphere (BSA concentration 1.45×10^{-7} mol/L) and a silane-coupled anti-BSA-coated surface. For each experiment, we added 1 μL of buffer solution (containing free BSA at various concentrations) to the chamber containing the microspheres in 100

μL of buffer and incubated this for 2 h at room temperature. We then measured the binding force of the microspheres to the anti-BSA-coated surface as described earlier. Fig. 3 shows the results of our experiments as a function of free BSA in solution.

The data in Fig. 3 indicate that the range of sensitivity of our assay covers at least three orders of magnitude. We observe an increase in the binding force of the microsphere to the surface for concentrations of BSA in solution between 1.45×10^{-12} and 1.45×10^{-15} mol/L. The difference in the binding force of the microsphere to the surface for 0 BSA in solution and for 1.45×10^{-15} mol/L BSA in solution shows that the assay is sensitive to femtomolar concentrations of antigens. At BSA concentrations of 1.45×10^{-11} mol/L and higher in solution, the binding force of the microsphere to the anti-BSA-coated surface is indistinguishable from the binding force of the microsphere to a silanized surface without anti-BSA (the NaB value in Fig. 3). We interpret this as arising from a complete displacement of the microsphere-coupled BSA–anti-BSA reaction by the free BSA. An upper limit for the average surface density of anti-BSA can be estimated, based on the minimum concentration for complete displacement. Because a concentration of 1.45×10^{-11} mol/L of BSA in solution corresponds to $\sim 9 \times 10^8$ molecules, the maximum average surface density of anti-BSA is 9×10^8 molecules/cm^2. However, this value is an overestimation because, in equilibrium, some fraction of the BSA is not bound but in solution. According to the data in Fig. 2, the increase in the difference between specific and nonspecific binding force for BSA concentrations of 1.45×10^{-12} and 1.45×10^{-7} mol/L is only threefold, even though the BSA surface coverage of the microsphere has increased by 10^5. This implies that the number of BSA to anti-BSA bonds is limited by the surface coverage of anti-BSA on the silanized coverslip and is at least threefold higher than the lower estimate based on the results with the nonspecific mouse IgG. We conservatively estimate the average surface density of anti-BSA to be between 6×10^7 and 9×10^8 molecules/cm^2.

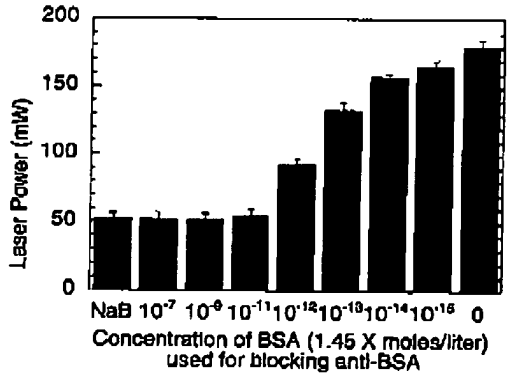

Fig. 3. Power required to pull microspheres coated with BSA (at 1.45×10^{-7} mol/L) off of a silane-coupled anti-BSA-coated glass cover slip in the presence of free BSA in solution.

The increase in the measured laser power corresponds to increasing binding force of the BSA-coated microspheres with the silanized surface. The decrease in the binding force with increasing free BSA concentrations is interpreted as arising from the decreasing number of anti-BSA binding sites available to the BSA-coated microsphere because of displacement by the free BSA in solution. The BSA concentrations indicated are those of free BSA in the chamber. Shown are the mean and SD of 10 measurements made with independent microspheres. *NaB*, measurements made with BSA-coated microspheres and a surface coated only with silane (no anti-BSA).

At a concentration of 1.45×10^{-15} mol/L, only $\sim 10^5$ molecules of BSA are in solution. A possible explanation for the detection of such a low concentration of analyte is that the dielectrophoretic force *[6]* of the laser acting on the free BSA molecules can effectively concentrate the free BSA in the region of contact between the microsphere of interest and the surface. In the presence of a gradient electric field, the interaction of the induced dipole moment of a molecule with the field results in a dielectrophoretic force on the molecule—essentially the same force responsible for trapping the microspheres by the optical tweezers. A simple calculation shows that, in the presence of the dielectrophoretic force of the laser field used in these experiments, the time required for a free BSA molecule (with a diffusion constant of 0.59 cm^2/s) to diffuse a distance of 100 μm (approximately the average distance between free BSA molecules at 1.45×10^{-15} mol/L) to the focus of the laser beam is close to the timescale in which we make our measurements (5 s). The polarizability of the glass coverslip should also enhance the electric field at the point through which the laser beam passes and increase the dielectrophoretic force at the location where the microsphere binds to the surface, thereby further concentrating the free BSA. Although our simple estimate of the timescale for bringing the free BSA molecules to a location where they could bind to anti-BSA and block the microsphere from sticking is reasonable, a more detailed calculation would include the binding of the BSA to the anti-BSA. Such a calculation, however, is beyond the scope of this paper, but further studies of this interesting dielectrophoretic effect are warranted.

In conclusion, we have demonstrated an optical tweezers-based immunosensor capable of detecting femtomolar concentrations of antigen in a competitive-binding assay. Although we have shown that the system described is capable of such highly sensitive measurements, an investigation of the limits of sensitivity as well as the development of some form of sample processing and microfluidics will be necessary for maximum utilization of this technology in the clinical environment. We believe the sensitivity of this technology can be improved further than has been demonstrated here. The broad potential of this approach for detection and quantification of binding pairs is under further investigation.

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